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# THE USE OF CHINESE HAMSTER OVARY CELLS TO QUANTIFY SPECIFIC LOCUS MUTATION AND TO DETERMINE MUTAGENICITY OF CHEMICALS

## A REPORT OF THE GENE-TOX PROGRAM [1]

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*Abbreviations*: AG, 8-azaguanine, CHO cells, Chinese hamster ovary cells; FBS, fetal bovine serum, HGPRT, hypoxanthine—guanine phosphoribosyl transferase; TG, 6-thioguanine(2-amino-6-mercaptopurine).

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## Summary

The GENE-TOX Group on Specific Gene Mutations in Chinese Hamster Ovary (CHO) Cells has evaluated the use of mutational systems in these cells for identification of mutagenic chemicals from 261 references in the file of the Environmental Mutagen Information Center, Oak Ridge National Laboratory by February, 1979; 68 references were found to be relevant to the stated task. After establishing that the end-point of mutational measurement occurs at a specific locus and the determinations are quantifiable and reproducible, data from 21 references were found to fulfill such requirements. Among them, 14 were concerned with chemically-induced mutations to resistance to a purine analogue, 6-thioguanine, which selects for variants deficient in the enzyme hypoxanthine—guanine phosphoribosyl transferase (HGPRT). This mutational system is referred to as the CHO/HGPRT assay. Studies with other genetic markers offer promise for the development of quantitative specific gene-mutational assays, but these studies have not advanced thoroughly enough to assess their value.

Several lines of genetic, physiological and biochemical evidence support the premise that the CHO/HGPRT system fulfills the criteria for measurement of specific gene mutations using CHO-$K_1$-$BH_4$ subclone and other appropriate CHO subclones. Based largely on published information, this Work Group has suggested a protocol for testing of chemical agents with consideration of the

following: cells, media, culture conditions and their quality control, treatment with test compounds with and without an exogenous metabolic activation system, estimation of cytoxicity (cloning efficiency), optimum expression and selection of the mutant phenotype, calculation of mutation frequency, positive and negative controls, vehicles or solvents, spontaneous mutation frequency, dosage selection and number of doses, and collection of raw data.

For interpretation of the mutagenesis data, this Work Group recommends various ways of presenting data, numerous criteria for acceptability of data, the need to use appropriate statistical procedures for data evaluation, and a potential applicability of results to hazard evaluation.

Evaluation of test performances with 18 chemicals revealed that the correlation between mutagenicity in CHO/HGPRT assay and animal mutagenicity and carcinogenicity is high. Since the number of chemicals tested was small and 17 of the 18 compounds were direct-acting agents, the utility of the system for identification of various classes of potential mutagens and carcinogens cannot be adequately assessed until more chemical classes, especially promutagens, are tested. However, the assay has a sound genetic and biochemical basis for quantifying specific locus mutation reproducibly. The fact that CHO cells are also useful for determination of chemically-induced chromosome aberration and sister-chromatid exchange adds an additional strength to the assay. Future research should address the possible improvement of procedures for phenotypic expression and application for testing gaseous and volatile liquids, as well as such problems as appropriate metabolic activation system(s) and effective statistical procedures common to perhaps all short-term cellular assays. Recent rapid development of mutagen test systems like the CHO/HGPRT assay calls for a need to update and evaluate the data base generated.

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## I. Introduction

As part of the GENE-TOX Program of the U.S. Environmental Protection Agency, a work group was formed to evaluate the use of Chinese hamster ovary (CHO) cells in culture, for identification of mutagenic chemicals. The task of this work group was to establish criteria for quantitative induction of specific locus mutations, to evaluate the current status of the mutational systems by summarizing the mutagenic activity of chemicals tested that meet these criteria, to propose a protocol for general use, and to recommend areas for further research and development.

We concurred that any assay utilizing mammalian cells must provide methodology to demonstrate that the end-point of the measurement is a mutational event which occurs at a *specific locus* and that the determinations must be *quantitative* and *reproducible.*

The evidence that a mutational event has been induced should be consistent with a heritable alteration in the DNA sequence of a gene. The variant phenotype must be shown to be stable in the absence of the selective condition. For demonstration of phenotypic stability, a number of variant clones should be randomly chosen, grown in non-selective medium for a length of time judged adequate in consideration of the altered phenotype, then tested for retention

of the original phenotype under conditions identical to the original selective conditions. In addition, evidence for an altered, specific genotype should be presented. In view of the difficulty in demonstrating an altered DNA sequence, biochemical identification of an altered gene product, such as altered enzyme activity or altered protein structure in stable variant clones, should be documented. Since the stated goal of this study is the demonstration of single gene mutation, the great majority of mutational events in a given system should be shown to reside in a specific gene, as, for example, the measurement of mutation to a specific auxotrophic requirement vs. auxotrophy in general, or to a specific temperature-sensitive phenotype vs. general undefined temperature sensitivity. This requires that alterations in specific gene products be demonstrated by biochemical measurements, chromosome location, complementation analyses, and similar methodology.

CHO cells have been widely used in studies of somatic cell genetics, biochemistry, biophysics, molecular biology, physiology and cellular biology for over two decades. Recent genetic studies have been performed with either the CHO line, which contains 21 chromosomes, or its derivative, CHO-$K_1$ line, which has 20 chromosomes (Kao and Puck, 1968). The cells are perhaps the best characterized mammalian cells genetically and are readily synchronized by various physical and chemical means. They exhibit a routine cloning efficiency higher than 80% in a reasonably well-defined medium on a glass or plastic substratum or in suspension, with a population doubling time of 12—14 h (Hsie et al., 1975; Kao and Puck, 1968). The CHO-$K_1$-$BH_4$ subclone, which has a relatively low spontaneous mutation frequency at the hypoxanthine—guanine phosphoribosyl transferase (HGPRT, *hgprt*) locus, has been used extensively for studying mutagen-induced cytotoxicity and gene mutation (Hsie et al., 1975). The growth properties, physiological characteristics and chromosomal constitution of this subclone are indistinguishable from its parental CHO-$K_1$ cells (Hsie et al., 1975; Kao and Puck, 1968). The relatively stable and easily recognizable karyotype has made the CHO cell line and its derivatives one of the best choices for studies on the effects of mutagens at the chromosome and chromatid level as well as for studies of gene mutation and cytotoxicity.

A literature search conducted by the Environmental Mutagen Information Center at Oak Ridge National Laboratory in February, 1979, (Search No. 769, under the subject of CHO cells) cited 261 publications dealing with the interactions of chemicals with the genetic apparatus of CHO cells. 56 references were abstracts or symposia papers which were outside the scope of this evaluation (Appendix 1). Of the remaining 206 references, 12 were concerned only with determination of cytotoxicity (Appendix 2), 16 with DNA damage and repair (Appendix 3), 52 with cytogenetic effects (Appendix 4), and 58 with parameters such as cell growth, DNA synthesis, and cell division (Appendix 5). The remaining 67 references addressed the subject of gene mutation to varying degrees.

To fulfill the stated task, this work group established the following criteria for evaluating these 68 references.

(1) The end-point measured in the assay must represent a mutational event occurring at a specific locus. Data supporting a mutation include heritability of the variant phenotype in the absence of selective conditions and biochemical

evidence of an altered gene product. In addition, it is important to demonstrate that the majority of mutations giving rise to the altered phenotype result from damage to a specific gene. For example, if temperature sensitivity is measured, evidence should be presented regarding the specific gene associated with the sensitivity.

(2) The experiments must be conducted in a way that would allow a quantitative response to be observed. More than one concentration of test chemical must be tested. Parameters such as phenotypic expression time and conditions for the selection of the variant phenotype, including concentration of the selective agent, selection medium and cell density, should be adequately defined to allow the maximal recovery of mutant cells.

(3) The experiments must be reproducible, that is, similar observations must be made in separate experiments.

With these considerations as a basis, the data in the majority of the studies concerned with gene mutation did not meet the criteria established for this evaluation. Most studies were not intended or designed to quantify mutation induction at specific genes. Instead, they dealt with the isolation and characterization of spontaneous or chemically induced phenotypic variants, new methods to induce novel altered phenotypes, and the the development of new mutational assays for mutagenesis studies. Some of the genetic markers studied include auxotrophic variants (Chang et al., 1977; Chasin et al., 1974; Hankinson, 1976; Jones and Moore, 1976; Jones and Puck, 1973; Kao, 1974; Kao and Puck, 1972, 1974; Patterson, 1976; Patterson and Carnright, 1977; Puck and Kao 1967; Taylor et al., 1971) enzyme deficiency (Stamato and Hohmann, 1975; Stamato and Jones, 1977) resistance to antimetabolites (Baker et al., 1974; Caboche and Mulsant, 1978; Chasin, 1974; Flintoff et al., 1976a, b; Gupta and Taylor, 1978; Gupta and Siminovitch, 1976, 1978; Jones and Sargent, 1974; Poeche et al., 1975a, b; Taylor et al., 1977; Whitfield et al., 1978) and conditional lethal variants such as temperature sensitivity (Adair et al., 1978; Farber and Unrau, 1975, 1976; Schroeder and Hsie, 1975; Thompson and Lindl, 1976; Thompson et al., 1978). These genetic markers offer promise for the development of quantitative gene mutation assays, but they have not been studied thoroughly enough to assess their value as quantitative gene mutation assays. Specifically, the use of resistance to ouabain, 8-azaadenine, 2,6-diaminopurine, emetine, $\alpha$-amanitin, cycloheximide and methotrexate, and the reversion of temperature-sensitive tRNA synthetase mutants must be developed further to allow adequate evaluation of their utility as reliable assays to identify chemical mutagens. Each of the studies in which these end-points are measured suffers from one or more deficiencies, such as a lack of data on phenotypic expression time, little or no evidence for specific locus alterations, and only one concentration of mutagen was tested in many cases.

A number of studies have employed the induction of nutritional auxotrophs as a measure of induction of gene mutation. However, these studies usually employed only one concentration of the test chemical and did not supply information on the specific locus nature of the induced variants (Kao and Puck, 1968, 1969, 1971, 1974; Patterson et al., 1974). This was also true for several reversion studies of mutant cells (Farber and Deutscher, 1976; Huberman and Sachs, 1976; Molnar and Rauth, 1976; Thompson et al., 1973). There were

also 3 references which were concerned with gene complementation, linkage and segregation (Chasin, 1972; Harris and Whitmore, 1977; Kao et al., 1969), and 1 which was a review article with no original data (Parodi and Brambilla, 1977).

22 references remained which fulfilled the established criteria. All of these were concerned with the use of purine analogues to select for resistant variants deficient in the enzyme HGPRT. Of these, 8 were studies of the conditions necessary for the isolation of variant clones or of the induction of variant clones by non-chemical agents (Carson et al., 1974; Hsie et al., 1977b; Jostes et al., 1977, 1978; Riddle and Hsie, 1978; Rosenstraus and Chasin, 1975; Taylor et al., 1978; Theile et al., 1976). The remaining 14 references (Aebersold, 1976; Carrano et al., 1978; Chasin, 1973; Couch et al., 1978; Couch and Hsie, 1978; Hsie et al., 1975, 1977a, 1978; O'Neill et al., 1977a, b, 1978; O'Neill and Hsie, 1977, 1979; Rasko et al., 1976) were then evaluated to judge the utility of CHO cells and 6-thioguanine (TG) resistance as a methodology for testing the mutagenicity of chemicals. This mutational system is referred to as the CHO/HGPRT assay.

This report consists of 4 sections: a description of the test system and the information which suggests that the end-point has determined induction of specific locus mutation quantitatively; a discussion of data interpretation; an analysis of test performance with various chemicals; and, an evaluation of the utility of the assay for mutagen screening.

## II. Test description

### *A. Genetic basis of the effect detected*

The frequencies of spontaneous and induced mutations are measured at the *hgprt* locus in CHO cells. The CHO/HGPRT assay was designed to select for variant cells resistant to such purine analogues as TG and 8-azaguanine (AG) as a result of mutation at the *hgprt* locus. The HGPRT enzyme utilizes the substrates 5-phosphoribosyl-1-pyrrophosphate and hypoxanthine or guanine to catalyze the formation of inosine- or guanosine-monophosphate, and thus con contribute to purine biosynthesis through the so-called salvage pathway. The purine analogues TG and AG are also substrates for this enzyme, and the mutant selection is based on the fact that wild-type mammalian cells containing HGPRT activity are capable of converting these purine analogs to the corresponding nucleotides which are then lethal to the cells. The presumptive mutants, deficient in HGPRT activity, are unable to form the lethal nucleotides and, thus, would grow in the presence of these analogs. It is known that the toxicities of nucleotides formed from TG and AG probably result from incorporation into DNA and RNA, respectively, or may be due to effects on the purine biosynthetic pathways.

The CHO/HGPRT system appears to fulfill criteria consistent with the premise that specific gene mutations are being measured. These criteria include: (1) A spontaneous mutation frequency in the range of $10^{-6}$—$10^{-5}$ mutants/cell. This frequency is similar to frequencies observed in other mutation assays in which there is evidence for mutational events. (2) Mutation by physical and chemical agents which follow linear concentration—response relationships. (3)

A frequency of spontaneous reversion in the range of $10^{-8}$—$10^{-7}$ revertants/cell. The reversion frequency would be expected to be lower than the forward mutation frequency because reversion involves a specific type of mutation. (4) The failure to observe TG-resistant cells in a near-tetraploid population (Chasin, 1973; Hsie et al., 1977a) since the presence of 2 copies of the functional hgprt genes would substantially decrease the frequency of mutagen-induced HGPRT-deficient cells. (5) Reduced HGPRT enzyme activity in approximately 98% of a large number of the TG-resistant clones tested (Hsie et al., 1977a; O'Neill et al., 1977a).

*B. Description of cells used*

In the majority of studies performed with the *hgprt* locus in which quantitative induction of mutation was demonstrated, the CHO-$K_1$-$BH_4$ subclone was used (Hsie et al., 1975). Other subclones of CHO cells are appropriate if (1) they exhibit a stable spontaneous mutation frequency, (2) they are sensitive to mutagens in a concentration-dependent fashion, (3) the optimal phenotypic expression time is established, and (4) evidence is presented to demonstrate the great majority of representative TG-resistant cells are HGPRT-deficient. However, the use of a single strain by different investigators eliminates the concern that subclone differences are responsible for variable results.

The CHO-$K_1$-$BH_4$ cell line is a proline auxotroph with a modal chromosome number of 20. Under the published growth conditions, in monolayer culture in Ham's F12 medium (Kao and Puck, 1968) containing 5—10% fetal bovine serum (FBS) (heat-inactivated and either undialyzed or dialyzed), the population doubling time has been stated to be 12—14 h and the cloning efficiency to be usually greater than 80% (Hsie et al., 1975).

*C. Suggested protocol for testing of chemical agents*

This protocol has been developed largely from the literature cited (Couch et al., 1978; Couch and Hsie, 1978; Hsie et al., 1975, 1977a, b, 1978; O'Neill et al., 1977a, b, 1978; O'Neill and Hsie, 1977, 1979; Riddle and Hsie, 1978), with some input based on the experience and judgment of the work group. Because of this, the methodology in the suggested protocol does not precisely conform to the literature evaluated. Since future research may suggest ways in which some aspects of the procedure may be improved, the protocol outlined here is subject to modification; we recommend that any modification should be documented before it is included in an established protocol.

*1. Cells*

The CHO-$K_1$-$BH_4$ subclone or other CHO cells have been shown to be appropriate for use. Criteria for considering the suitability of other subclones should include stability of karyotype, responsiveness to mutagens, etc., as discussed earlier. Cells should be routinely checked for mycoplasma contamination, karyotypic stability, and genetic markers.

*2. Medium*

Ham's medium F12 (Kao and Puck, 1968), which was designed originally for clonal growth of CHO cells, has been prepared by rehydration of commer-

cially available powder. The liquid medium has been used most frequently at a slightly alkaline condition by adjusting to pH 7.2—7.5 prior to the addition of sodium bicarbonate or serum. Stock cultures are grown in F12 medium or hypoxanthine-free F12 medium containing 10% heat-inactivated (56°, 30 min) FBS. No antibiotics are used. For selecting TG-resistant mutants, hypoxanthine-free F12 medium containing 5—10% heat-inactivated FBS is used. All other portions of experiments are performed with F12 medium, with or without hypoxanthine containing 5—10% heat-inactivated FBS. The antibiotics penicillin (50 units/ml) and streptomycin (50 μg/ml) may be used in growth media and in experiments. It has also been demonstrated that hypoxanthine-free F12 medium (also available commercially in powder) containing 5—10% dialyzed FBS can be used for all phases of experiments and cultivation of stock cultures since CHO-$K_1$ cells do not require hypoxanthine for growth (Kao and Puck, 1968).

*3. Cell culture conditions*

The cultures are maintained in exponential growth by subculturing every 2—3 days at 37°C in an atmosphere of 95—100% relative humidity and 5% $CO_2$ in air. For cell detachment to subculture, 0.05% trypsin has usually been employed.

*4. Treatment with test compounds*

The protocol for treatment of cells with chemicals has been developed for monolayer cultures. Since CHO cells also grow in suspension culture, it might be useful to use such cultures in testing protocols. However, no definite procedure has been published for mutagen testing in suspension cultures and, therefore, it is inappropriate at present to recommend a test procedure using a suspension culture for mutagen treatment.

Exponentially growing cultures are plated at a density of $5 \times 10^5$ cells/25-$cm^2$ flask (or other appropriate vessel) in Ham's F12 medium containing 5% dialyzed FBS. These cultures are then incubated 16—24 h to allow cell attachment and growth to $\sim 0.9—1.2 \times 10^6$ cells/vessel. The time of initiation of treatment with the chemical mutagen, for the purpose of this discussion, is designated as day 0.

Experiments are conducted both in the presence and in the absence of an exogenous activation system. In the absence of an activation system, the treatment medium consists of F12 medium with or without hypoxanthine containing 5—10% dialyzed heat-inactivated FBS and test chemical delivered in 20—60 μl. The treatment medium may be buffered with Hepes buffer (0.025 M, pH 7.2). The cells are incubated in the presence of the treatment medium of 3—5 ml for 16—24 h, which is over one population doubling time of CHO cells. Highly reactive chemicals may exhibit mutagenic activity for only a small portion of this treatment time. For this reason and for the convenience of comparing the mutagenic activity in the presence of an activation system with a treatment time of 5 h, it becomes practical to expose cells with test chemical with or without activation for 5 h. Serum is generally included in the treatment medium to support normal cell growth during this time. However, serum should be omitted during treatment if the chemicals being tested are known to

be inactivated by serum or to bind to serum with a high affinity.

Experiments must also be conducted in the presence of an activation system because CHO cells lack the enzymes necessary to metabolize many promutagens to their mutagenic forms. Published procedures with CHO cells include the use of either a host-mediated assay (Hsie et al., 1978) or a rat-liver homogenate fraction (O'Neill et al., 1977b), the so-called S9 preparation. The choice of these or other activation methods will depend on the nature of the chemical and the requirements of the investigator. No single activation system has proved to be optimal for testing all chemicals. However, a general guideline for using the activation system is presented. A major consideration is whether the rats from which livers are obtained should be pretreated with an inducer to enhance the enzyme levels involved in metabolizing chemicals to the mutagenic forms since no single inducer is necessarily optimal for all chemicals. However, an appropriate starting point would be to use livers from 8—9 week old rats pretreated with Aroclor 1254 at 500 mg/kg 5 days before sacrifice. The livers are homogenized in 0.15 M KCl or a phosphate buffer at pH 7.2. The homogenate is centrifuged at $9000 \times g$ for 15 min at 4°C. After the concentration of protein is determined, the S9 fraction is stored in liquid nitrogen until use. The treatment medium of 3—5 ml for conducting experiments with activation is composed of F12 medium with or without hypoxanthine, S9, Hepes buffer ($2.5 \times 10^{-2}$ M, pH 7.2), magnesium chloride ($1 \times 10^{-3}$ M), glucose 6-phosphate ($4.7 \times 10^{-3}$ M), NADP ($1.5 \times 10^{-3}$ M), and test chemical added in a volume of 20—60 $\mu$l for 5 h. This treatment period is chosen because the enzymes in the S9 do not remain fully functional beyond 5 h, and the high S9 concentrations used may be toxic if treatment lasts longer than that.

The conditions specified for treating CHO cells both with and without activation are known to provide reproducible and reliable results (O'Neill et al., 1977a, b). However, it should be stressed that these conditions are not necessarily the best conditions for all chemicals under all circumstances; thus it is important to establish the particular conditions that may be most relevant for the chemical being tested. The most appropriate inducer and amount of S9 protein may also be different between different chemicals.

Cells treated with or without an activation system are washed after the 5-h or other appropriate incubation period and incubated in F12 medium with or without hypoxanthine containing 5—10% heat-inactivated dialyzed FBS for an additional 18—24 h. At this time, the cells are subcultured to assess cytotoxicity and to begin the phenotypic expression period.

*5. Estimation of cytotoxicity*

For cytotoxicity determination, the cells are tested for 5 h or other appropriate time, removed from the vessel with trypsin (0.02—0.05%), and diluted to appropriate cell concentrations; aliquots of 200—5000 cells (depending on the expected cell survival) are plated in 60-mm dishes in 5 ml of medium F12 containing 5% dialyzed FBS (usually 3 or more dishes per treatment point are plated for cytotoxicity estimation). After 7—8 days incubation, the colonies are fixed, stained and counted. A cluster of more than 50 cells growing within a confined area is considered a colony. Cytotoxicity is usually expressed relative to the untreated control culture, whose absolute cloning efficiency should be given.

*6. Expression of the mutant phenotype*

The expression of the TG-resistant phenotype has been shown to be maximal by 7—9 days after treatment and to remain steady thereafter for all mutagens tested thus far (O'Neill et al., 1977a, 1978; O'Neill and Hsie, 1977, 1979; Riddle and Hsie, 1978). Subculture of approx. $10^6$ cells at 48-h intervals normally has been employed, i.e., subculture on days 1, 3 and 5 and selection on day 7, or subculture on days 1, 3, 5 and 7 and selection on day 9. Other subculture regimens have been employed without altering the optimum expression of the mutant phenotype. Investigators whose purposes necessitate an alternative procedure should provide a demonstration that optimal phenotypic expression has occurred.

*7. Selection of the mutant phenotype*

The use of hypoxanthine-free medium F12 containing 5% dialyzed FBS and 10 $\mu$M TG has been shown to select efficiently colonies of cells with a highly reduced HGPRT activity (Hsie et al., 1975, 1977a; O'Neill et al., 1977a). Investigators using other media or selective agents or other concentrations of TG should demonstrate the suitability of these conditions. AG is not recommended for this purpose since this purine analogue is relatively unstable under the selection condition and it requires medium change during the selection period (Jostes et al., 1977, 1978). Cloning efficiency of the cells prior to mutant selection is determined in the same medium without TG.

The density of cells in the selection medium and other factors such as $CO_2$ level can affect the optimum recovery of mutants under selective conditions. The pH and volume of the medium apparently also have effects. A density of $2 \times 10^5$ cells/100-mm dish in 10 ml of medium has been routinely employed for the selection procedure. For cloning efficiency determinations, 200 cells/60-mm dish in 4—5 ml of medium is used.

After 7—8 days of incubation, the colonies are fixed, stained and counted for both mutant selection and cloning efficiency determination.

*8. Calculation of mutation frequency*

The mutation frequency is calculated by dividing the total number of mutant colonies by the number of cells selected (usually $10^6$ cells: 5 plates at $2 \times 10^5$ cells/plate), corrected for the cloning efficiency of cells prior to mutant selection, and is expressed as mutants per $10^6$ clonable cells or per $10^6$ survivors.

*9. Positive and negative controls*

The choice of controls depends on the chemical being tested. An attempt should be made to use a positive control chemical that is structurally similar to the test chemical. When structurally similar positive controls are not available, ethyl methanesulfonate is an appropriate control for direct-acting mutagen (without an activation system), and 7,12-dimethylbenz[*a*]anthracene, benzo-[*a*]pyrene, dimethylnitrosamine or 2-acetylaminofluorene are appropriate for promutagens (with an activation system).

*10. Vehicles or solvents*

The choice of an appropriate solvent depends on the physical properties of the test chemical. Commonly used solvents include water, dimethylsulfoxide, ethanol and acetone. The effect or activity of the solvent in the assay must be clearly defined. The investigator should determine the potential cytotoxic and mutagenic effects of the solvent at the concentrations used and the possible impairment to the function of the activation system. Dimethylsulfoxide is the organic solvent of choice since dimethylsulfoxide even at a relatively high concentration does not inactivate the S9 system. A solvent control should be included in each experiment.

*11. Spontaneous mutation frequency*

The approximate range of spontaneous mutation frequencies reported in experiments performed according to the method described is 0—20 mutants per $10^6$ clonable cells. When these experimental conditions are used, spontaneous mutation frequencies in this range are considered acceptable.

A sound basis for deciding upon a spontaneous mutation frequency range which is universally appropriate for different cell systems, different markers, and different experimental conditions does not exist. An acceptable spontaneous mutation frequency for a given experimental condition, therefore, can only be defined empirically with a sound scientific basis.

*12. Dosage selection and number of doses*

An attempt should be made to test a minimum of 3 concentrations of the test chemical that gives cell survivals of approximately 100, 50 and 10% survival relative to control cell survival. Survival lower than 10% might prevent an accurate determination of a low induced mutation frequency, since a high cellular lethality would greatly reduce the number of the total surviving treated cell population. Documentation of cytotoxic effect is especially useful to demonstrate an effect on the cells if no mutagenic activity is observed. Data from the same 3 concentrations should be obtained in duplicate experiments. Acceptable reasons for conducting experiments with a maximum concentration that does not give the desired degree of cytotoxicity include (1) the test chemical cannot be dissolved at a high enough concentration in an appropriate solvent to be cytotoxic, and (2) precipitation of the test chemical or test chemical dissolved in solvent upon addition to treatment medium is excessive or causes an unusual cytotoxicity.

*13. Collection of raw data*

Each piece of raw data, e.g., the number of surviving and mutant colonies per plate used to determine cytotoxicity and mutation frequency respectively, should be recorded following good laboratory practices. Raw data should not be averaged, transformed, or corrected before recording.

**III. Interpretation of data**

*A. Presentation of data*

*1. Tables and graphs*

Data on mutation frequency and cytotoxic effect can be presented in either figures (e.g., concentration—response curves) or tables, depending on the

requirement of the investigator, and should be in a manner which permits easy critical evaluation by the reader. The number of replicates performed and the range of observed values or standard deviations should be indicated. Although the investigator can freely choose to present data in the form of either tables or figures depending on the point being made, tabular data can be better evaluated since the reported number can be directly used; estimation from a figure by a reader could create unnecessary errors. Original data on mutant number and cytotoxicity of each replicate are desirable.

*2. Data transformation*

Data transformation is acceptable if it is necessary to demonstrate a point. A clear description of the method used for transformation and the reasons for its appropriateness for use on the data should be presented. The presentation should include sufficient information to allow the reader to transform the data back to its original state.

*3. Various units of expression*

Mutation data should be expressed as "TG-resistant mutants/$10^6$ clonable cells". For the purpose of comparing activities of different chemicals or sensitivities of systems, an expression of data which incorporates concentration, length of treatment, and degree of activity would be useful.

*B. Criteria for acceptability of data*

(1) Properties of the cell line used, characteristics of cell growth, and experimental conditions should be clearly described, especially when related to phenotypic expression procedure and selection conditions. (2) Good laboratory practices should be adhered to. (3) Actual data or a means to reconstruct original data should be presented. (4) Results from all controls and 3 concentrations of the test chemical should be presented. (5) The observations should be shown to be reproducible. (6) The range and average of spontaneous mutation frequencies (or the average with 95% confidence limits) observed in the laboratory during the period preceding the experiments of interest should be presented. (7) Information regarding the purity of the test chemical should be presented. This may range from a statement that purity was unknown to a detailed analysis. Information on purity strengthens the conclusions drawn. (8) Appropriate positive control chemicals should be used for experiments conducted in the presence and absence of a metabolic activation system. (9) Negative controls may provide useful information for interpreting the meaning of data but should not be required. Preferably, negative controls should use non-mutagenic/carcinogenic chemicals, structurally related to the test chemicals. Solvent controls and untreated cultures should always be performed in all experiments. (10) Consideration should be given to the appropriateness of the activation system used, including: (a) The use of an inducer for the induction of mixed-function oxidase activity and the appropriateness for the type of chemical tested; (b) An evaluation of the effect of tissue homogenate or feeder-cell concentration on test chemical activity; and (c) The appropriateness of the source (species, tissue) of the activation system for the test chemical or chemical class. More research is required in all these areas.

*C. Statistical evaluation*

There is a need to develop statistical methods to objectively and uniformly determine whether test chemicals exhibit mutagenic activity. Although statistical methods are being proposed, a single method has not yet been agreed upon. One method involves a direct data fitting to a linear model (Hsie et al., 1975), and the other involves data transformation prior to model fitting (J.D. Irr and R.D. Snee, personal communications), followed by an analysis of the difference of the slope of the dose–response curves between the control and experimental groups. Studies of mathematical theories associated with the CHO/HGPRT assay for the development of an effective statistical procedure, especially with a low mutagenic response, are encouraged. Such studies should take into consideration the intrinsic characteristics of the assay protocol from which numerous factors are expected to affect quantitative mutagenesis.

*D. Criteria for positive/negative conclusions*

Data should be reproducible regardless of the responses. Positive compounds should exhibit a concentration-related increase over at least 3 concentrations.

Criteria for determining whether a chemical gives a positive or a negative response in the CHO assay cannot be developed without taking into consideration the variability of the spontaneous mutation frequencies and the frequencies observed in the presence of the test chemical. The only methods thus far proposed and used for analyzing CHO test data are cited in Section C. An effective and reliable statistical method, which takes into consideration various factors characteristic of the assay, should be used to draw positive and negative conclusion.

*E. Applicability of results to hazard evaluation*

The data obtained from this assay indicate a likelihood or potential of the test chemical to be a mutagen or carcinogen for humans. Since a direct correlation between mutagenicity in the CHO/HGPRT assay and in animals or humans is not fully established, the data are not a basis for classifying chemicals either as animal or human mutagens/carcinogens or as nonhazardous. Likewise, the information cannot be used to establish acceptable exposure levels. In addition, the information presently available does not provide a basis for estimating the potency of a chemical as a mutagen or carcinogen in animals or humans on the basis of the degree of mutagenic activity.

## IV. Test performance

The adequacy of testing the mutagenic activity of chemicals in the CHO/HGPRT assay was evaluated from 14 references described in Section I.

The recommendation that at least 3 concentrations of a chemical be tested in duplicate experiments was not met in some of these cases (Couch et al., 1978; Couch and Hsie, 1978; Hsie et al., 1978; O'Neill et al., 1977a, b). In addition, 3 of the references could not be evaluated at all, since in 2 of these only one concentration of the chemical was employed (Chasin, 1973; Rasko et al., 1976), and in the third all treatment with the test chemical was performed in the presence of another suspected mutagen, 5-bromodeoxyuridine (Carrano

TABLE 1

INTERRELATIONSHIPS OF CARCINOGENICITY AND MUTAGENICITY IN ANIMALS AND MUTAGENICITY IN CHO/HGPRT ASSAY

| Chemical (CAS Registry No.) | Animal carcinogenicity [a] | Animal mutagenicity [b] | Mutagenic activity [a] in CHO/HGPRT (lowest effective concentration [c], μM) |
|---|---|---|---|
| *N*-nitroso compounds | | | |
| Nitrosamines | | | |
| Dimethylnitrosamine (62-75-9) | + | + | + [d] (<30) |
| Nitrosamides | | | |
| *N*-Methyl-*N*-nitrosourea (684-93-5) | + | | + (<15) |
| *N*-Ethyl-*N*-nitrosourea (759-73-9) | + | | + (<300) |
| *N*-Butyl-*N*-nitrosourea (869-01-2) | + | | + (<1000) |
| Nitrosamidines | | | |
| *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (70-25-7) | + | | + (<0.2) |
| *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine (4275-77-6) | + | | + (<1.0) |
| Alkane sulfonates | | | |
| Methyl methanesulfonate (66-27-3) | + | + | + (<50) |
| Ethyl methanesulfonate (62-50-0) | + | + | + (<200) |
| Isopropyl methanesulfonate (926-06-7) | + | | + (<1500) |
| Alkyl sulfates | | | |
| Dimethyl sulfate (77-78-1) | + | | + (<50) |
| Diethyl sulfate (64-67-5) | + | | + (<750) |

| | | |
|---|---|---|
| Heterocyclic nitrogen compounds | | |
| ICR 170 (146-59-8) | + | + (<0.25) |
| ICR 170-OH (35591-47-0) | — | — (negative) |
| ICR 191 (17070-44-9) | + | + (<0.25) |
| ICR 191-OH (38915-18-3) | — | — (negative) |
| ICR 292 (4251-89-2) | + | + (<0.1) |
| ICR 372 (1798-09-3) | + | + (<0.75) |
| Metallic compounds | | |
| *cis*-Dichlorodiamine platinum (II) (15663-27-1) | + | + (<2.0) |

[a] Data on carcinogenicity of *N*-nitroso compounds, alkane sulfonate, and alkyl sulfates were based on either IARC Monograph on Evaluation of Carcinogenic Risk of Chemicals to Man, Vols. 1—10 (1972—76) IARC, Lyon; or USPHS Publication 149, Survey of Compounds which have been Tested for Carcinogenic Activity (1967—73), USPHS, Bethesda. Data on heterocyclic nitrogen compounds came from Peck et al. (1976), Cancer Res. 36, 2423, and ibid, J. Med. Chem., 19, 1422; data on *cis*-dichlorodiammine platinum (II) came from Leopold et al. (1979) Cancer Res., 39, 913.

[b] Data on animal mutagenicity were based on a report from Comparative Mutagenesis Conference National Institute of Environmental Health Sciences, Research Triangle Park, October 30—November 5, 1977, on Induction of Specific Locus Mutation in Mice by U.H. Ehling. The mammalian Spot Assay by R. Frahrig and G.W.P. Dawson, or The Heritable Translocation Test in Mammals by W. Generoso, B. Cattanach and A.H. Malashenko.

[c] The values of lowest effective concentration are the concentrations resulting in a mutation frequency of $50 \times 10^{-6}$ mutants/clonable cell. This value is considered reasonable based on the generally observed spontaneous mutation frequency ($0—20 \times 10^{-6}$ mutants/clonable cell) and average replicate standard deviation (±25%). Since the actual spontaneous mutation frequency and the replicate standard deviation must be used to calculate the lowest mutation frequency that is statistically higher than the spontaneous mutation frequency observed in the concurrent experiment, the $5 \times 10^{-6}$ value itself is generally not acceptable for determining whether a chemical is mutagenic or not. These calculations could not be performed because the standard deviation were not presented and, in some cases, the spontaneous mutation frequency could not be accurately estimated from graphic presentations of data in the papers evaluated by this Work Group.

[d] Dimethylnitrosamine is not mutagenic S9. All other chemicals were assayed without S9.

et al., 1978) which complicates the interpretation of the mutagenicity of test chemical per se. In 2 of these studies there were no data on phenotypic expression time of the mutant phenotype (Carrano et al., 1978; Rasko et al., 1976). Another study contained no data on the spontaneous mutation frequency (Aebersold, 1976). 10 references (Couch et al., 1978; Couch and Hsie, 1978; Hsie et al., 1975, 1977a, 1978; O'Neill et al., 1977a, b, 1978; O'Neill and Hsie, 1977, 1979) in general were deficient in that they were intended not as reports of mutagen testing but as reports of the development of a system suitable for testing and comparative studies of the activity of known mutagens in that system.

Data on the mutagenicity of 18 chemical compounds are available from these references: 6 *N*-nitroso compounds, 3 alkane sulfonates, 2 alkyl sulfates, 6 heterocyclic nitrogen compounds, and 1 metallic compound. (3 additional chemicals, 5-bromodeoxyuridine, mitomycin C and proflavin, were included in the references judged unsuitable for evaluation). Of these, 17 are direct-acting agents (listed in Table 1). Dimethylnitrosamine, which requires a metabolic activation system to exert cytotoxicity and mutagenicity, has been tested, utilizing both the host (mouse) mediated assay and rat-liver S9. Of the 18 chemicals, 16 were positive and 2 were negative in this mutagenicity assay.

The correlations between animal mutagenicity and carcinogenicity studies are shown in Table 1. The correlation seems to be high, although the number of chemicals tested is small and 17 of the 18 compounds are direct-acting agents.

The published data do not provide enough information to assess adequately the utility of the CHO/HGPRT assay in identifying various classes of potential mutagens and carcinogens.

## V. Conclusions

### *A. Strengths of the assay*

The system utilizing CHO cells and TG resistance to measure mutation induction appears to be a sensitive assay, at least with the compounds which have been reported. Although these are mostly alkylating agents, structure—activity relationship has been demonstrated. The assay, performed according to the standard protocol, provides reproducible results with a high sensitivity upon examination of data published from different laboratories at different times. Parameters such as phenotypic expression time and selection conditions are defined, allowing a quantification of the mutagenic activity of the chemicals tested. The assay has a sound genetic and biochemical basis and thus has potential for mechanism studies. A reasonably standardized and defined protocol has been developed and shown to be reproducible with a limited number of chemicals.

Other well-documented advantages include the well known fact that CHO cells have also been used to detect other end-points such as chromosome/chromatid aberrations and sister-chromatid exchanges. The cells can also be cultivated as monolayer or suspension cultures and are readily synchronized by several methods.

In addition, the CHO/HGPRT mutational system has a human equivalent, i.e., the Lesch—Nyhan syndrome, an X-linked recessive disease resulting from a

deficiency of HGPRT activity. It has been estimated that 40—50% of reported cases arise by new mutations likely through environmental influence. Mutational events occurring in the CHO/HGPRT system and human diseases can thus be correlated.

*B. Weaknesses of the assay*

The system has largely been used with direct-acting agents. Other classes of chemicals, especially those requiring metabolic activation, need to be used before general utility of this system can be assessed. The relatively long phenotypic expression time, according to the proposed protocol, somewhat limits the number of tests which can be performed within a defined period of time.

*C. Role in testing programs*

This assay could play a role in mutagenicity testing programs since it appears to be sensitive and allows quantification. Its role in predicting carcinogenicity cannot be judged until more chemicals have been tested and the correlations with animal carcinogenicity have been developed. Even then the problems inherent in the use of in vitro tests, as well as mutagenicity tests, apply. There is a potential advantage in that it is a mammalian cell test system capable of determining multiple distinct genetic end-points.

*D. Recommendations*

The results from mutagenicity testing of more chemical classes are required to allow a thorough evaluation of the sensitivity of the CHO/HGPRT system. These should include various major classes of mutagens and carcinogens and their non-mutagenic and non-carcinogenic structural analogues. Much work has already been done but is not yet published.

Efforts have been initiated and should be continued to determine whether a continued cell growth with frequent subculturing during the phenotypic expression time is necessary. If the mutant phenotype can be fully expressed without any subculturing, the assay will become less time-consuming and less costly.

The metabolic activation system represents the weakest aspect of this assay. This is true for other short-term mutation assays as well. Work to establish the proper activation conditions or methods to rapidly decide which conditions are appropriate needs to be continued. Some factors requiring consideration are the proper species and inducer to use for tissue homogenate preparation, the proper concentration(s) of tissue homogenate to use for testing chemicals, and whether intact cells should be used rather than tissue homogenates.

With this and other systems, methods are required for testing gases and volatile liquids, since chemicals having these physical properties comprise a significant portion of the chemicals which may be tested. A method for testing gaseous mutagens in the CHO/HGPRT assay is being developed (D.F. Krahn, personal communication).

Work to develop and refine statistical methods for clearly delineating mutagenicity vs. non-mutagenicity as determined by the CHO/HGPRT assay, as well as by other mutagen testing systems, must be encouraged. A method applied to the CHO/HGPRT system is being developed (personal communi-

cations from J.D. Irr and R.D. Snee; and W.Y. Tan and A.W. Hsie).

Evaluations of various test systems covered under the GENE—TOX Program should be conducted frequently in light of the rapid development of systems like the CHO/HGPRT assay. To the best knowledge of this work group, there are more published data on compounds being tested for mutagenicity using this assay during 1979—80 than the entire evaluation period (1968—79) covered by the present task.

## References

Adair, G.M., L.H. Thompson and P.A. Lindl (1978) Somatic Cell Genet., 4, 27—44.
Aebersold, P.M. (1976) Mutation Res., 36, 357—362.
Baker, R.M., D.M. Brunette, R. Mankovitz, L.H. Thompson, G.F. Whitmore, L. Siminovitch and J.E. Till (1974) Cell, 1, 9—21.
Caboche, M., and P. Mulsant (1978) Somatic Cell Genet., 4, 407—421.
Carrano, A.V., L.H. Thompson, P.A. Lindl and J.L. Minkler (1978), Nature (London), 271, 551—553.
Carson, M.P., D. Vernick and J. Morrow (1974) Mutation Res., 24, 47—54.
Chang, T.Y., C. Tekalowski, W. van den Heuvel, A.W. Alberts and P.R. Vagelos (1977) Proc. Natl. Acad. Sci. (U.S.A.), 74, 832—836.
Chasin, L.A. (1972) Nature (London), New Biol., 240, 50—52.
Chasin, L.A. (1973) J. Cell Physiol., 82, 299—308.
Chasin, L.A. (1974) Cell, 2, 37—41.
Chasin, L.A., A. Feldman, M. Konstam and G. Urlaub (1974) Proc. Natl. Acad. Sci. (U.S.A.), 71, 718—722.
Couch, D.B., and A.W. Hsie (1978) Mutation Res., 57, 209—216.
Couch, D.B., N.L. Forbes and A.W. Hsie (1978) Mutation Res., 57, 217—224.
Farber, R.A., and M.P. Deutscher (1976) Somatic Cell Genet., 2, 509—520.
Farber, R.A., and P. Unrau (1975) Mol. Gen. Genet., 138, 233—242.
Farber, R.A., and P. Unrau (1976) Methods Cell Biol., 14, 265—271.
Flintoff, W.F., S.V. Davidson and L. Siminovitch (1976a) Somatic Cell Genet., 2, 245—261.
Flintoff, W.F., S.M. Spindler and L. Siminovitch (1976b) In Vitro, 12, 749—757.
Gupta, K.C., and M.W. Taylor (1978) Mutation Res., 49, 95—101.
Gupta, R.S., and L. Siminovitch (1976) Cell, 9, 213—219.
Gupta, R.S., and L. Siminovitch (1978) Somatic Cell Genet., 4, 77—93.
Hankinson, O. (1976) Somatic Cell Genet., 2, 497—507.
Harris, J.F., and G.F. Whitmore (1977) Somatic Cell Genet., 3, 173—193.
Hsie, A.W., P.A. Brimer, T.J. Mitchell and D.G. Gosslee (1975) Somatic Cell Genet., 1, 247—261.
Hsie, A.W., P.A. Brimer, R. Machanoff and M.H. Hsie (1977a) Mutation Res., 45, 271—282.
Hsie, A.W., A.P. Li and R. Machanoff (1977b) Mutation Res., 45, 333—342.
Hsie, A.W., R. Machanoff, D.B. Couch and J.M. Holland (1978) Mutation Res., 51, 77—84.
Huberman, E., and L. Sachs (1976) Proc. Natl. Acad. Sci. (U.S.A.), 73, 188—192.
Jones, C., and E.E. Moore (1976) Somatic Cell Genet., 2, 235—243.
Jones, C., and T.T. Puck (1973) J. Cell Physiol., 81, 299—304.
Jones, G.E., and P.A. Sargent (1974) Cell, 2, 43—54.
Jostes, R.F., W.C. Dewey and L.E. Hopwood (1977) Mutation Res., 42, 139—144.
Jostes, R.F., L.E. Hopwood, W.C. Dewey and G.R. Blackburn (1978) Mutation Res., 50, 433—440.
Kao, F.T. (1974) Biochem. Dis., 4, 565—574.
Kao, F.T., and T.T. Puck (1968) Proc. Natl. Acad. Sci. (U.S.A.), 60, 1275—1281.
Kao, F.T., and T.T. Puck (1969) J. Cell. Physiol., 74, 245—258.
Kao, F.T., and T.T. Puck (1971) J. Cell. Physiol., 78, 139—144.
Kao, F.T., and T.T. Puck (1972) J. Cell. Physiol., 80, 41—50.
Kao, F.T., and T.T. Puck (1974) Methods Cell Biol., 8, 23—39.
Kao, F.T., L. Chasin and T.T. Puck (1969) Proc. Natl. Acad. Sci. (U.S.A.), 64, 1284—1291.
Molnar, S.J., and A.M. Rauth (1976) Mutation Res., 41, 361—376.
O'Neill, J.P., and A.W. Hsie (1977) Nature (London), 269, 815—817.
O'Neill, J.P., and A.W. Hsie (1979) Mutation Res., 59, 109—118.
O'Neill, J.P., P.A. Brimer, R. Machanoff, G.P. Hirsch and A.W. Hsie (1977a) Mutation Res., 45, 91—101.
O'Neill, J.P., D.B. Couch, R. Machanoff, J.R. San Sebastian, P.A. Brimer and A.W. Hsie (1977b) Mutation Res., 45, 103—109.
O'Neill, J.P., J.C. Fuscoe and A.W. Hsie (1978) Cancer Res., 38, 506—509.

Parodi, S., and G. Brambilla, (1977) Mutation Res., 47, 53—74.
Patterson, D. (1976) Somatic Cell Genet., 2, 189—203.
Patterson, D., and D.V. Carnright (1977) Somatic Cell Genet., 3, 483—495.
Patterson, D., F.T. Kao and T.T. Puck (1974) Proc. Natl. Acad. Sci. (U.S.A.), 71, 2057—2061.
Poeche, H., N.B. Varshaver and E. Geissler (1975a) Mutation Res., 27, 399—406.
Poeche, H., N.B. Varshaver, M. Theile and E. Geissler (1975b) Mutation Res., 30, 83—88.
Puck, T.T., and F.T. Kao (1967) Proc. Natl. Acad. Sci. (U.S.A.), 58, 1227—1234.
Rasko, I., K. Burg and L. Dallmann (1976) Theor. Appl. Genet., 48, 157—162.
Riddle, J.C., and A.W. Hsie (1978) Mutation Res., 52, 409—420.
Rosenstraus, M., and L.A. Chasin (1975) Proc. Natl. Acad. Sci. (U.S.A.), 72, 493—497.
Schroeder, C.H., and A.W. Hsie (1975) Exp. Cell Res., 91, 170—174.
Stamato, T.D., and L.K. Hohmann (1975) Cytogenet. Cell Genet., 15, 372—379.
Stamato, T.D., and C. Jones (1977) Somatic Cell Genet., 3, 639—647.
Taylor, M.W., J.H. Pipkorn, M.K. Tokito and R.O. Pozzatti Jr. (1977) Somatic Cell Genet., 3, 195—206.
Taylor, M.W., M. Souhrada and J. McCall (1971) Science, 172, 162—163.
Taylor, M.W., M.K. Tokito, K.C. Gupta and J. Pipkorn (1978) Biochim. Biophys. Acta, 517, 1—13.
Theile, M., S. Scherneck and E. Geissler (1976) Mutation Res., 37, 111—124.
Thompson, L.H., and P.A. Lindl (1976) Somatic Cell Genet., 2, 387—400.
Thompson, L.H., J.L. Harkins and C.P. Stanners (1973) Proc. Natl. Acad. Sci. (U.S.A.), 70, 3094—3098.
Thompson, L.H., D.J. Lofgren and G.M. Adair (1978) Somatic Cell Genet., 4, 423—435.
Whitfield, C.D., B. Buchsbaum, R. Bostedor and E.H.Y. Chu (1978) Somatic Cell Genet., 4, 699—713.

## Appendix 1. Abstracts and symposia papers

Adair, G.M., L.H. Thompson and P.A. Lindl (1977) J. Cell Biol., 75, 382A.
Baker, M.S., W.N. Bell, D.S. Greenberg, D.G. Kaufman and J.W. Grisham (1977) In Vitro, 13, 184.
Baker, R.M., and R. Mankovitz (1973) Genetics, 74, S15.
Barranco, S.C., and R.M. Humphrey (1970) Int. Cancer Congr. Abstr., 10, 433.
Borman, L.S., J.N. Dumont and A.W. Hsie (1973) J. Cell Biol., 59, 32A.
Brown, R.F. (1978) Fed. Proc., 37, 234.
Burki, H.J. (1977) Radiat. Res., 70, 650.
Cancer Testing Technology and Saccharin (1977) Office of Technology Assessment, U.S. Congress, U.S. Government Printing Office, Washington, D.C., 149 pp.
Carrano, A.V., L.H. Thompson, J.L. Minkler and P.L. Lindl (1978) Mutation Res., 53, 162—163.
Carver, J.H., D.L. Wandres, G.M. Adair and E.W. Branscomb (1978) Mutation Res., 53, 96—97.
Chasin, L.A. (1973) J. Cell Biol., 59, 50A.
Chasin, L.A., M. Rosenstraus and G. Urlaub (1975) in: Cell Biology and Tumor Immunology, Excerpta Med. Int. Congr. Ser., 349 (Vol. 1) 52—55.
Clark, E.P., and J.T. Lett (1976) Radiat. Res., 67, 519.
Couch, D.B., and A.W. Hsie (1976) Mutation Res., 38, 399.
Coutino, R., J. Meyne and M.S. Legator (1978) Mutation Res., 53, 80.
Dean, B.J., D.H. Hudson, A.S. Wright and D.E. Stevenson (1978) Pharmacologist, 20, 154.
Deaven, L.L., E.W. Campbell and S.S. Barham (1977) Mamm. Chromosomes Newsl., 18 (1/2) 17.
Douglas, G.R., and C.E. Grant (1978) Can. J. Genet. Cytol., 20, 442.
Epler, J.L., and A.W. Hsie (1978) ORNL-5375, pp. 48—60.
Fishbein, L. (1976) IARC (Int. Agency Res. Cancer) Sci. Publ., 12, 41—59.
Fogu, G., A. Mottura, G. Della Valle and E. Calendi (1977) Atti Assoc. Genet. Ital., 22, 163—164.
Friedman, M.A., E.J. Greene, J.A. Sherrod and A.J. Salerno (1978) Pharmacologist, 20, 154.
Hodgson, J.R., M.A. Kowalski, J.P. Glennon, J.C. Dacre and C.-C. Lee (1976) Mutation Res., 38, 387.
Hsie, A.W., and P.A. Brimer (1975) Genetics, 80, S44.
Hsie, A.W., and T.T. Puck (1972) J. Cell Biol., 55, 118A.
Hsie, A.W., P.A. Brimer, T.J. Mitchell and D.G. Gosslee (1975) Mutation Res., 31, 322—323.
Hsie, A.W., P.A. Brimer, J.P. O'Neill and D.B. Couch (1975) J. Cell Biol., 67, 182A.
Hsie, A.W., D.B. Couch, P.A. Brimer, J.P. O'Neill and R. Machanoff (1977) Proc. Am. Assoc. Cancer Res., 18, 160.
Hsie, A.W., D.B. Couch, J.R. San Sebastian, W.N.C. Sun, J.C. Riddle, P.A. Brimer, R. Machanoff, N.L. Forbes, F.T. Kennedy and M.H. Hsie (1978) Proc. Am. Assoc. Cancer Res., 19, 93.
Hsie, A.W., R. Machanoff, D.B. Couch and J.M. Holland (1978) Mutation Res., 53, 94.
Ikushima, R. (1974) Senshokutai (Kromosomo) 96/97, 3014.
Ikushima, T. (1977) J. Radiat. Res., 18, 19.
Kao, F.T., and T.T. Puck (1975) Genetics, 79, 343—352.
Kedinger, C., and R. Simard (1973) J. Cell Biol., 59, 163A.
Kimler, B.F., and M.H. Schneiderman (1977) Proc. Am. Assoc. Cancer Res., 18, 11.

Leeper, D.B., B.F. Kimler and M.H. Schneiderman (1976) Radiat. Res., 67, 585.
Masubuchi, M., S. Nawai, M. Hirokado and K. Hiraga (1978) Mutation Res., 54, 242—243.
Meyn, R.E., and R.M. Humphrey (1970) Int. Cancer Congr. Abstr., 10, 349.
Molnar, S.J., L.H. Thompson and A.M. Rauth (1976) Can. J. Genet. Cytol., 18, 567.
Natarajan, A.T. (1977) Mutation Res., 46, 144.
Pipkorn, J., M. Tokito and M.W. Taylor (1976) In Vitro, 12, 307—308.
Rao, P.N., and S. Mahagaokar (1975) Proc. Am. Assoc. Cancer Res., 16, 74.
Rossman, T., D. Lustig and W. Troll (1977) In Vitro, 13, 201.
Siciliano, M.J., and R.M. Humphrey (1975) Mutation Res., 31, 323.
Siciliano, M.J., and R.M. Humphrey (1976a) J. Cell Biol., 70, 204A.
Siciliano, M.J., and R.M. Humphrey (1976b) Mamm. Chromosomes Newsl., 17 (1/2) 63.
Siciliano, M.J., and R.M. Humphrey (1978) Mutation Res., 53, 115—116.
Sincock, A.M. (1976) Origins Hum. Cancer, Cold Spring Harbor Symp., 39.
Sunkara, P.S., P.N. Rao and K. Nishioka (1977) Proc. Am. Assoc. Cancer Res., 18, 84.
Takehisa, S., and S. Wolff (1978) Mutation Res., 53, 77.
Taylor, M.W., G. Chinchar, M. Tokito and K. Gupta (1977) In Vitro, 13, 195.
Tomasovic, S.P., and W.C. Dewey (1976) Radiat. Res., 67, 631.
Tsuda, H., K. Kato, T. Matsumoto, D. Yoshida and S. Mizusaki (1978) Mutation Res., 54, 260.
Vogel, E., and A.T. Natarajan (1978) Mutation Res., 53, 279—280.
Waldren, C.A. (1973) Diss. Abstr. Int. B. Sci. Eng., 34, 553—554.
Yoshida, S., M. Masubuchi and K. Hiraga (1978) Mutation Res., 54, 262.

## Appendix 2. Studies of cytotoxicity

Barranco, S.C., and D.R. Fluornoy (1977) J. Natl. Cancer Inst., 58, 657—663.
Bhambhani, R., J. Kuspira and R.E. Giblak (1973) Can. J. Genet. Cytol., 15, 605—618.
Busse, P.M., S.K. Bose, R.W. Jones and L.J. Tolmach (1977) Radiat. Res., 71, 666—677.
Chadwick, K.H., H.P. Leenhouts, I. Szumiel and A.H.W. Nias (1976) Int. J. Radiat. Biol., 30, 511—524.
Dewey, W.C., L.E. Stone, H.H. Miller and R.E. Giblak (1971) Radiat. Res., 47, 672—688.
Fox, M. (1974) Mutation Res., 24, 187—204.
Fox, M., C.W. Gilbert, L.G. Lajtha and A.H.W. Nias (1970) Chem.-Biol. Interact., 1, 241—246.
Kasschau, M.R., J. Elder and R.E. Meyn (1977) Chem.-Biol. Interact., 18, 205—215.
Nias, A.H.W., and B.W. Fox (1976) Chem.-Biol. Interact., 14, 93—99.
Nias, A.H.W., and I.I. Szumiel (1977) J. Clin. Hematol. Oncol., 7, 562—568.
Szumiel, I., and A.H.W. Nias (1976) Br. J. Cancer, 33, 450—458.
Voiculelz, N., K.C. Smith and H.S. Kaplan (1974) Cancer Res., 34, 1038—1044.

## Appendix 3. Studies of DNA damage and repair

Barranco, S.C., J.K. Novak and R.M. Humphrey (1975) Cancer Res., 35, 1194—1204.
Clarkson, J.M. (1978) Mutation Res., 52, 273—284.
Clarkson, J.M., and R.M. Humphrey (1976) Cancer Res., 36, 2345—2349.
Corry, P.M., S. Robinson and S. Getz (1977) Radiology, 123, 475—482.
Darzynkiewicz, Z., F. Traganos, T. Sharpless and M.R. Melamed (1977) Exp. Cell Res., 110, 201—214.
Gautschi, J.R., B.R. Young and R.B. Painter (1972) Biochim. Biophys. Acta, 281, 324—328.
Gautschi, J.R., B.R. Young and J.E. Cleaver (1973) Exp. Cell Res., 76, 87—94.
Hahn, G.M., S.J. Yang and V. Parker (1968) Nature (London), 220, 1142—1144.
Harris, J.W., C.J. Koch, J.A. Power and J.E. Biaglow (1977) Radiat. Res., 70, 585—596.
Klamerth, O.L. (1978) Mutation Res., 50, 251—261.
Koerner, I., W. Malz and C. Pitra (1976) Biol. Zentralbl., 95, 561—577.
Miller, C.T., P.D. Lawley and S.A. Shah (1973) Biochem. J., 136, 387—393.
Moore, P.D., and R. Holliday (1976) Cell, 8, 573—579.
Palcic, B., and L.D. Skarsgard (1978) Br. J. Cancer (Suppl. III), 37, 54—59.
Van der Schans, G.P., H.B. Centen and P.H.M. Lohman (1979) Mutation Res., 59, 119—122.
Walters, R.A., R.A. Tobey and C.E. Hildebrand (1976) Biochim. Biophys. Acta, 447, 36—44.

## Appendix 4. Cytogenetic studies

Alam, M.T., M. Corbeil, A. Chagnon and S.S. Kasatiya (1974) Chromosoma, 49, 77—86.
Alam, M.T., N. Barthakur, N.G. Lambert and S.S. Kasatiya (1978) Can. J. Genet. Cytol., 20, 23—30.
Ashwood-Smith, M.J., and G.B. Friedmann (1979) Cryobiology, 16, 132—140.

Au, W., M.A. Butler, S.E. Bloom and T.S. Matney (1979) Mutation Res., 66, 103—112.
Au, W., S. Pathak, C.J. Collie and T.S. Hsu (1978) Mutation Res., 58, 269—276.
Barrau, M.D., G.R. Blackburn and W.C. Dewey (1978) Cancer Res., 38, 2290—2294.
Cox, D.M., and T.T. Puck (1969) Cytogenetics, 8, 158—169.
Deaven, L.L., and D.F. Petersen (1973) J. Cell Biol., 59, 73A.
Deaven, L.L., and R.A. Tobey (1977) J. Cell Biol., 75, 10A.
Deaven, L.L., M.S. Oka and R.A. Tobey (1978) J. Natl. Cancer Inst., 60, 1155—1161.
De Raat, W.K. (1977) Chem.-Biol. Interact., 19, 125—131.
Dewey, W.C., and H.H. Miller (1971) Exp. Cell Res., 66, 283—288.
Dewey, W.C., and L.E. Stone (1975) Exp. Cell Res., 94, 299—309.
Dewey, W.C., A. Westra, H.H. Miller and H. Nagasawa (1971) Int. J. Radiat. Biol., 20, 505—520.
Evans, H.J., and P. Perry (1977) Mutation Res., 46, 116.
Hittleman, W.N., and P.N. Rao (1973) J. Cell Biol., 59, 144A.
Hittleman, W.N., and P.N. Rao (1974a) Mutation Res., 23, 251—258.
Hittleman, W.N., and P.N. Rao (1974b) Mutation Res., 23, 259—266.
Hittleman, W.N., and P.N. Rao (1974c) Cancer Res., 34, 3433—3439.
Hittleman, W.N., and P.N. Rao (1975) Cancer Res., 35, 3027—3035.
Hsu, T.C., C.J. Collie, A.F. Lusby and D.A. Johnston (1977) Mutation Res., 45, 233—247.
Ikushima, T. (1977) Nature (London), 268, 235—236.
Ikushima, T., and S. Wolff (1974) Exp. Cell Res., 87, 15—19.
Korenberg, J.R., and E.F. Freedlender (1976) Mamm. Chromosomes Newsl., 17(4), 19—22.
Latt, S.A., and K.S. Loveday (1978) Cytogenet. Cell Genet., 21, 184—200.
Latt, S.A., S.H. Munroe, C. Disteche, W.E. Rogers and D.M. Cassell (1977) Chromosomes Today, 6, 27—36.
Lau, Y.F., R.L. Brown and F.E. Arrighi (1977) Exp. Cell Res., 110, 57—61.
Mace, M.L., Jr., Y. Daskal and W. Wray (1978) Mutation Res., 52, 199—206.
Maier, P., and W. Schmid (1976) Mutation Res., 40, 325—338.
McGill, M., S. Pathak and T.C. Hsu (1974) Chromosoma, 47, 157—167.
Natarajan, A.T., and G. Obe (1978) Mutation Res., 52, 137—149.
Natarajan, A.T., A.D. Tates, P.P.W. van Buul, M. Meijers and N. de Vogel (1976) Mutation Res., 37, 83—90.
Obe, G., and H. Ristow (1977) Mutation Res., 56, 211—213.
Pal, K., B. Tierney, P.L. Grover and P. Sims (1978) Mutation Res., 50, 367—375.
Perry, P. (1977) Mutation Res., 46, 205.
Perry, P., and H.J. Evans (1975) Nature (London), 258, 121—125.
Perry, P.E., and C.E. Searle (1977) Mutation Res., 56, 207—210.
Rao, P.N., and A.P. Rao (1976) Proc. Am. Assoc. Cancer Res., 17, 86.
Rao, P.N., and R.T. Johnson (1972) J. Cell Sci., 10, 495—513.
Roszinsky-Köcher, G., and G. Röhrborn (1979) Mutation Res., 66, 199—203.
Sincock, A., and M. Seabright (1975) Nature (London), 257, 56—58.
Stetka, D.G., and S. Wolff (1976) Mutation Res., 41, 343—350.
Stetten, G., S.A. Latt and R.L. Davidson (1976) Somatic Cell Genet., 2, 285—290.
Stich, H.F., L. Wei and P. Lam (1978) Cancer Lett., 5, 199—204.
Szumiel, I., and A.H.W. Nias (1976) Chem.-Biol. Interact., 14, 217—232.
Takehisa, S., and S. Wolff (1977) Mutation Res., 45, 263—270.
Takehisa, S., and S. Wolff (1978) Mutation Res., 58, 103—106.
Wolff, S. (1977a) Annu. Rev. Genet., 11, 183—201.
Wolff, S. (1977b) in A. Castellani (Ed.), Research in Photobiology, Plenum, New York, pp. 721—732.
Wolff, S., and B. Rodin (1978) Science, 200, 543—545.
Wolff, S., and S. Takehisa (1977) Dev. Toxicol. Environ. Sci., 2, 193—200.
Wray, W., and S. Harrison (1975) J. Cell Biol., 67, 464A.

## Appendix 5. Cell physiology and regulation studies

Barham, S.S., and B.R. Brinkley (1974) J. Cell Biol., 63, 15A.
Barham, S.S., and B.R. Brinkley (1976a) Cytobios, 15, 85—96.
Barham, S.S., and B.R. Brinkley (1976b) Cytobios, 15, 97—109.
Barranco, S.C., and W.E. Bolton (1977) Cancer Res., 37, 2589—2591.
Barranco, S.C., and R.M. Humphrey (1970) Mutation Res., 9, 524—526.
Barranco, S.C., and R.M. Humphrey (1971a) Cancer Res., 31, 191—195.
Barranco, S.C., and R.M. Humphrey (1971b) Cancer Res., 31, 1218—1223.
Barranco, S.C., and R.M. Humphrey (1971c) Mutation Res., 11, 421—429.
Barranco, S.C., E.W. Gerner, K.H. Burk and R.M. Humphrey (1973) Cancer Res., 33, 11—16.

Boder, G.B. (1973) J. Cell Biol., 59, 28A.
Clarkson, J.M., and R.M. Humphrey (1977) Cancer Res., 37, 200—205.
Collins, A.R.S. (1977) Biochim. Biophys. Acta, 478, 461—473.
Cone Jr., C.D., and M. Tongier Jr. (1971) Oncology, 25, 168—182.
Cress, A.E., and E.W. Gerner (1977) Exp. Cell Res., 110, 347—353.
Dewey, W.C., and D.P. Highfield (1976) Radiat. Res., 65, 511—528.
Ehmann, U.K., and J.T. Lett (1972) Exp. Cell Res., 74, 9—14.
Everhart, L.P., and D.M. Prescott (1972) Exp. Cell Res., 75, 170—174.
Gautschi, J.R., and R.M. Kern (1973) Exp. Cell Res., 80, 15—26.
Gerulath, A.H., S.C. Barranco and R.M. Humphrey (1974) Cancer Res., 34, 1921—1925.
Guy, A.L., and J.H. Taylor (1978) Proc. Natl. Acad. Sci. (U.S.A.), 75, 6088—6092.
Highfield, D.P., and W.C. Dewey (1972) Exp. Cell Res., 75, 314—320.
Kimball, R.F., S.W. Perdue and A.W. Hsie (1975) Exp. Cell Res., 95, 416—424.
Kimler, B.F., and D.B. Leeper (1976) Cancer Res., 36, 3212—3216.
Leeper, D.B. (1973) J. Cell Biol., 59, 190A.
Ley, K.D., and R.A. Tobey (1970) J. Cell Biol., 47, 453—459.
Madoc-Jones, H., and F. Mauro (1974) Handb. Exp. Pharmakol., 38(1), 205—219.
McCarty Sr., K.S., N.R. Wallace, B. Kaufman and K.S. McCarty Jr. (1977) 35th Annu. Proc. Electron Microsc. Soc. Am., 35, 530—531.
McGill, M., D.P. Highfield, T.M. Monahan and B.R. Brinkley (1976) J. Ultrastruct. Res., 47, 43—53.
Meyn, R.E., R.R. Hewitt and R.M. Humphrey (1973) J. Cell Biol., 59, 224A.
Miller, D.R., W.C. Dewey and H.H. Miller (1973) Int. J. Radiat. Biol., 23, 591—602.
Miller, M.W., V. Ciaravino and G.E. Kaufman (1977) Radiat. Res., 71, 628—634.
Nagasawa, H., and W.C. Dewey (1972) J. Cell Physiol., 80, 89—106.
Ockey, C.H. (1972) Exp. Cell Res., 70, 203—213.
Ockey, C.H., and T.D. Allen (1975) Exp. Cell Res., 93, 275—282.
Poffenbarger, M., and G.M. Fuller (1976) Exp. Cell Res., 103, 135—141.
Ramseier, H.P., M. Burkhalter and J.R. Gautschi (1977) Exp. Cell Res., 105, 445—453.
Rao, A.P., and P.N. Rao (1976) J. Natl. Cancer Inst., 57, 1139—1143.
Roots, R., and K.C. Smith (1976) Cancer Res., 36, 3654—3658.
Schneiderman, M.H., W.C. Dewey and D.P. Highfield (1971) Exp. Cell Res., 67, 147—155.
Schneiderman, M.H., W.C. Dewey, D.B. Leeper and H. Nagasawa (1972) Exp. Cell Res., 74, 430—438.
Shuve, S.J., and A.W. Rauth (1971) Cancer Res., 31, 1422—1428.
Swartzendruber, D.E. (1977) Exp. Cell Res., 109, 439—443.
Sweeney, M.J., G.B. Boder, G.J. Cullinan, H.W. Culp, W.D. Daniels, R.W. Dyke, K. Gerzon, R.E. McMahon, R.L. Nelson, G.A. Poore and G.C. Todd (1978) Cancer Res., 38, 2886—2891.
Tobey, R.A. (1972a) Cancer Res., 32, 309—316.
Tobey, R.A. (1972b) Cancer Res., 32, 2720—2725.
Tobey, R.A. (1972c) J. Cell Physiol., 79, 259—265.
Tobey, R.A. (1975) Nature (London), 254, 245—247.
Tobey, R.A., and H.A. Crissman (1975) Cancer Res., 35, 460—470.
Tobey, R.A., and K.D. Ley (1970) J. Cell Biol., 46, 151—157.
Tobey, R.A., and K.D. Ley (1971) Cancer Res., 31, 46—51.
Tobey, R.A., D.F. Petersen, E.C. Anderson and T.T. Puck (1966) Biophys. J., 6, 567—581.
Tobey, R.A., M.S. Oka and H.A. Crissman (1975) Eur. J. Cancer, 11, 433—441.
Tobey, R.A., L.L. Deaven and M.S. Oka (1978) J. Natl. Cancer Inst., 60, 1147—1153.
Tolmach, L.J., R.W. Jones and P.M. Busse (1977) Radiat. Res., 71, 653—665.
Tomasovic, S.P., and W.C. Dewey (1978) Exp. Cell Res., 114, 277—284.
Walters, R.A., R.A. Tobey and C.E. Hildebrand (1976) Biochem. Biophys. Res. Commun., 69, 212—217.
Wheatley, D.N., and B. Angus (1973) Experientia, 29, 1393—1395.
Wheatley, D.N., and J.Y. Henderson (1974) Exp. Cell Res., 89, 431—436.